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RESEARCH ARTICLE

Genetic structure of natural populations of California red abalone (*Haliotis rufescens*) using multiple genetic markers

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Abstract Mitochondrial cytochrome oxidase subunit one (COI) sequence, nuclear microsatellites, and amplified fragment length polymorphisms (AFLPs) were used to evaluate connectivity among nine red abalone (*Haliotis rufescens*) populations sampled between August 1998 and November 2003 along approximately 1,300 km of California coastline from Crescent City (41°46′N, 124°12′W) to San Miguel Island (34°02′N, 120°22′W). COI sequences and microsatellite genotypes did not show significant genetic divergence among nine sampled populations. A subset of five populations spanning the geographic range of the study was scored for 163 polymorphic AFLP markers. Of these, 41 loci showed significant divergence ($P < 0.001$) among populations. Still, no AFLP markers were diagnostic for any of the study populations, and assignment tests did not consistently assign individuals to the correct population. Although the AFLP data are the first to suggest there is significant genetic differentiation among California red abalone populations, the discordance between the different genetic markers needs further study before unambiguous conclusions can be drawn with respect to connectivity among the populations.

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Introduction

Natural history considerations fail to make clear predictions regarding population connectivity in the red abalone (*Haliotis rufescens*), an ecologically important, broadcast spawning marine gastropod. Ocean currents suggest that dispersal among populations of this and other marine species may occur over long geographic distances (Waples 1998). Red abalone spawn from October through February in northern California and year-round in southern California (CDFG 2005), leaving their pelagic larvae subject to diverse coastal current systems. Even with a relatively short pelagic phase at 4–15 days, larvae entrained in the north-flowing Davidson Current of the California Current system could be transported at 0.2–0.3 m s^{-1} in January (Glickman 1999), theoretically traveling as far as 350 km, nearly one quarter of the California coastline. In addition, although red abalone larvae are competent to settle in 4 days at 18°C, an 8–18°C temperature range is tolerated (Leighton 2000), and cooler water temperatures (hence, longer dispersal times) are typical of winter months when the bulk of the species' spawning takes place.

Despite the potential for extensive dispersal, habitat characteristics and other early life-history traits favor local retention of abalone larvae. Adults are predominantly found in or near rocky macroalgal communities where water flow is attenuated by kelp (Gaylord et al. 2004), and abalone eggs are negatively buoyant (Moffet 1978; Leighton 2000). Furthermore, the potential for predation and starvation

(abalone larvae are lecithotrophic) increases and the probability of encountering suitable habitat decreases the longer the larvae remain in the plankton. Although little is known about the capabilities of larvae to select specific habitats, larval settlement occurs in response to chemical inducers such as the phycobilins produced by crustose coralline red algae (Morse et al. 1979; Morse and Morse 1984; Leighton 2000). Crustose corallines are found commonly in rocky low intertidal and subtidal zones that are often coincident with the macroalgal habitat where adult abalone are found, again suggesting a mechanism favoring local larval retention.

Past genetic research on the red abalone has indicated little or no differences in allelic frequencies among populations, consistent with high gene flow and exchange of migrants. Kirby et al. (1998) describe results for a single microsatellite locus genotyped in 74 individuals randomly selected from northern and southern California. Although the authors note that several rare alleles were unique to either the northern or southern California samples, no statistically significant genetic differences were observed. Burton and Tegner (2000) genotyped three allozyme loci and partially sequenced the mitochondrial cytochrome oxidase subunit one (COI) gene in samples from three California locales (two from northern California and one from San Miguel Island) to assess whether individuals from a 1979 outplanting of red abalone on San Miguel Island persisted through the following two decades as suggested by Gaffney et al. (1996). No genetic signal from the 1979 outplanting was evident, and allele frequencies and genic diversity did not differ among the samples.

We expand on the limited scope of previous work by analyzing the genetic connectivity among red abalone populations using a combination of nuclear and mitochondrial loci. We use mtDNA sequences, microsatellite polymorphisms, and amplified fragment length polymorphisms (AFLPs) to increase resolution of gene flow among red abalone populations. Geographically, our sample range starts near the Oregon, USA, border at Crescent City (41°46′N, 124°12′W), California, and spans approximately 1,300 km of California coastline, ending at San Miguel Island (34°02′N, 120°22′W) in the Southern California Bight. This encompasses over half of the species' range, which extends from northern California, USA, to central Baja California, Mexico (Geiger 2000).

Materials and methods

Detailed information on methods, including evidence of the mode of marker inheritance, is available in supplementary electronic appendix S1.

Samples and DNA extraction

Samples of red abalone tissue collected and archived between the years 1998 and 2003 from nine locations ($N = 474$) were obtained from the California Department of Fish and Game (CDFG) and L Rogers-Bennett at the University of California, Davis. Although abalone densities are >8,000 per hectare in some northern California locales (Rogers-Bennett et al. 2004), samples south of San Francisco Bay (SFB) are limited by low natural abundances. Collection sites, including sample sizes and site abbreviations, are shown in Figure 1 (see also S2 for sample collection information). From north to south along the California coast, sites included Crescent City, Trinidad Head, Shelter Cove, 7 House Cove near Caspar, Van Damme State Park, Salt Point State Park, Horseshoe Cove, Monterey, and San Miguel Island. A second independent sample of smaller abalone was obtained in 2001 from Van Damme State Park to examine sampling effects on genetic composition.

Cytochrome oxidase subunit one (COI)

A 580 basepair (bp) fragment of the mitochondrial COI gene was amplified from approximately 30 abalone per population ($N = 309$). Polymerase chain reaction (PCR) primers and cycling conditions were taken from Metz et al. (1998). A UPGMA dendrogram was generated in PAUP* 4.0b10 (Swofford 1998) and visualized with TREEVIEW (Page 1996) to define the COI haplotypes. To evaluate levels of genetic structure, exact tests of differentiation (Markov chain parameters: 1,000 dememorization steps, 10,000 permutations) and an hierarchical analysis of molecular variance (AMOVA 1,000 permutations) were performed in Arlequin v2.000 (Schneider et al. 2000) on the haploid COI sequence data. Significance of pairwise Φ_{ST} estimates was calculated based on χ^2 tests of the parameter value being significantly different from zero (i.e. no genetic divergence; $\chi^2 = 2N\Phi_{ST}$, $df = 1$). Results were sequential Bonferroni corrected at the $\alpha = 0.05$ level (Rice 1989). A COI haplotype network (Fig. 2) that takes into account both distance between haplotypes and haplotype frequency was generated using parsimony in the program TCS v1.18 (Clement et al. 2000). The hierarchical clades of the network were described topologically according to Templeton et al. (1995). Nested clade analysis (NCA) was performed on each cladistic level in order to find geographic structure among the haplotypes using the program GeoDis 2.2 (Posada et al. 2000).

Microsatellites

Genotyping was attempted at five microsatellite loci in all 474 red abalone. Basic information regarding the microsatellite loci is presented in Table 1. Loci included one

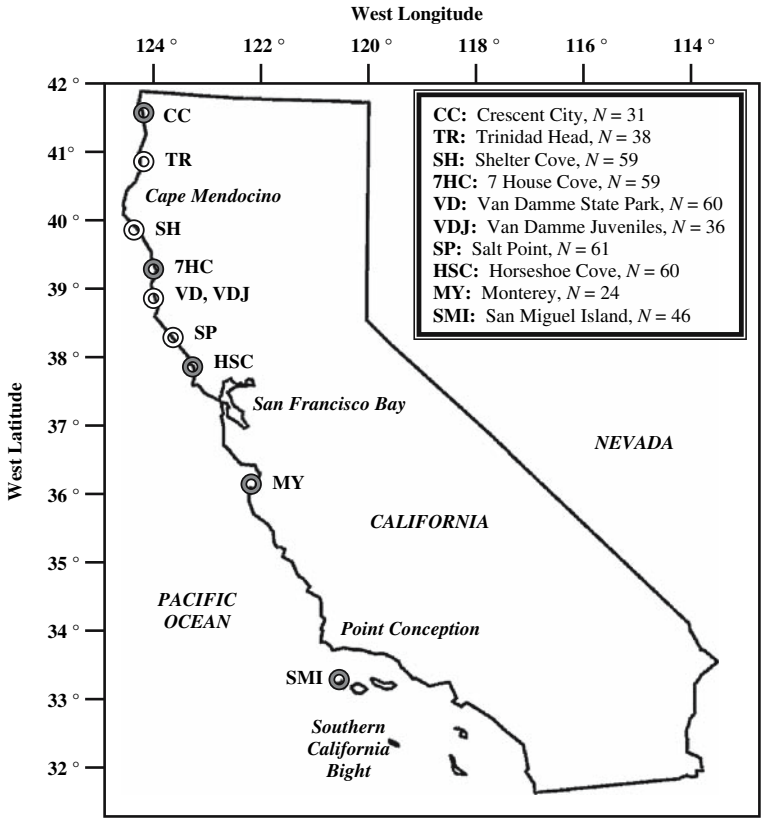

Fig. 1 Map of California collection sites for *H. rufescens*. Key includes sample size and abbreviation. *Filled circles* indicate population used in AFLP analyses

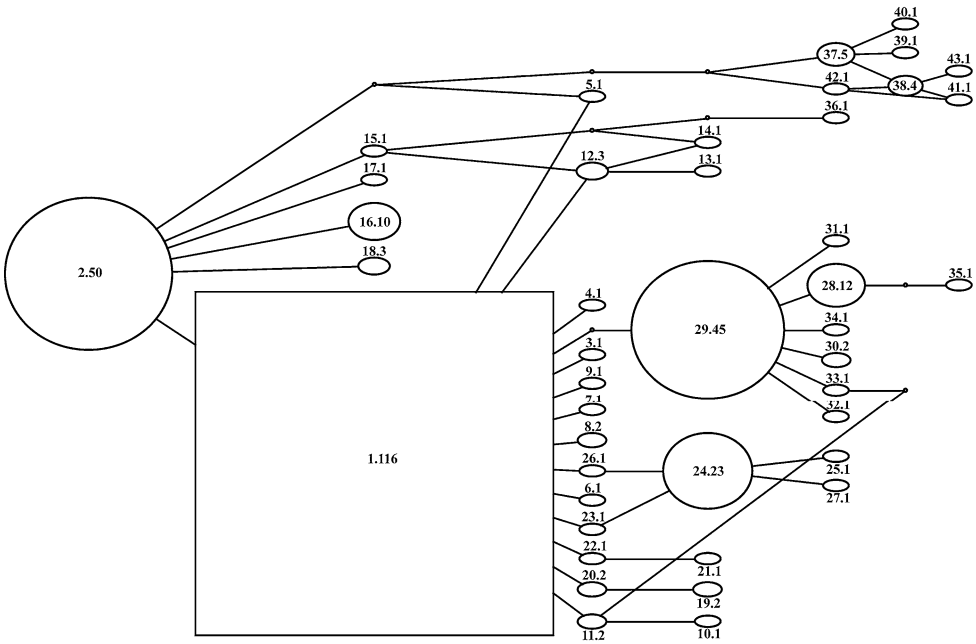

Fig. 2 COI haplotype network for *H. rufescens* from nine California sites. Highest frequency haplotype represented with a *square*; size of the *ovals* represents relative frequency of remaining haplotypes. *Ovals* labeled with haplotype names, including haplotype number, followed by a period and number of times that haplotype occurred. *Lines* represent single nucleotide substitutions. *Black dots* indicate missing intermediate haplotypes

compound tri-/tetranucleotide repeat (*Hka3*) and four dinucleotide repeats (*Hka*28, *Hka*40, *Hka*56, and *Hka*80). PCR primers used were originally developed for a related eastern Pacific species, the pinto abalone *H. kamtschatkana* (Miller et al. 2001). A maximum-likelihood estimate of the inferred frequency of the null allele was calculated for each locus using ML-NullFreq (Kalinowski and Taper 2006). Global and pairwise exact tests of genotypic differentiation (Markov Chain parameters: 10,000 dememorization steps, 1,000 batches, 10,000 permutations per batch) were performed in GENEPOP v3.4 (Raymond and Rousset 1995, 2003). GENEPOP was also used to calculate estimates of F_{ST} and the F_{ST} analogue ρ_{ST} (which takes into account microsatellite allele size assuming the stepwise mutation

Table 1 Descriptive statistics for nuclear microsatellite loci in *H. rufescens*

Locus	Repeat	Alleles	Individuals	H_e	H_o	*P* (SE)	Null
*Hka*3	$(GTA)_n(GAGT)_n$	75	445	0.96	0.90	0.06 (0.02)	0.03
*Hka*28	$(CA)_n$	29	429	0.88	0.86	0.67 (0.04)	0.01
*Hka*40	$(CA)_n$	28	448	0.91	0.91	0.60 (0.04)	0.00
*Hka*56	$(CA)_n$	26	401	0.80	0.42	0.00 (0.00)	0.31
*Hka*80	$(CA)_n$	36	396	0.83	0.73	0.00 (0.00)	0.06

Nucleotide repeat sequence, number of alleles, number of individuals genotyped out of 474 total (excludes null homozygotes), number of expected and observed heterozygotes, expected (H_e) and observed heterozygosities (H_o), Hardy–Weinberg equilibrium probability test *P*-values (*P*) with standard errors (SE) in parentheses, and null allele frequencies (Null) per locus are included

All microsatellite loci originally isolated in the northern abalone *H. kamtschatkana* (Miller et al. 2001)

model (Weir and Cockerham 1984; Slatkin 1995; Rousset 1996; Michalakis and Excoffier 1996)). Estimates of Φ_{ST} were calculated via AMOVA in Arlequin. Levels of significance were again calculated based on χ^2 tests of the parameter value being significantly different from zero ($\chi^2 = 2N\rho_{ST}$, $df = 1$), and results were sequential Bonferroni corrected at the $\alpha = 0.05$ level.

Amplified fragment length polymorphisms (AFLPs)

AFLP analyses were limited to a subset of 209 red abalone chosen to represent the full breadth of our geographic sampling effort; the full sample sets were included for CC, 7HC, HSC, MY, and SMI (minus 11 SMI DNAs that failed repeatedly in PCR; see also Fig. 1 for sample locations and abbreviations). Time constraints associated with scoring the allele banding patterns by hand prevented scoring AFLPs in all samples genotyped by mtDNA sequencing and microsatellites. Primer combinations giving strong smears of product in the appropriate size were chosen and are presented in Table 2. Protocols were performed according to Vos et al. (1995).

Initial calculations of divergence assumed HWE and were based on allele frequency differences; corrected allele frequencies were calculated in TFPGA v1.3 (Miller 1997) using the square root of the frequency of the null (recessive) genotype. TFPGA was also used to estimate per-locus global F_{ST} values (with jackknifing over the loci) and perform exact tests of global and pairwise differentiation (Markov Chain parameters: 2,000 dememorization steps, 20 batches, 5,000 permutations per batch). An AMOVA was performed in the Microsoft® Excel add-in program GenAlEx 6 (Peakall and Smouse 2006) to calculate estimates of the F_{ST} analogue Φ_{PT} ($\equiv\Phi_{ST}$). All results were sequential Bonferroni corrected at the $\alpha = 0.05$ level. A UPGMA cladogram was generated in TFPGA with 5,000 bootstrap replicates to determine the topological relationships among the five populations.

An outlier test of selection was conducted on our AFLP loci to establish whether the pattern of divergence seen in the AFLPs may be due primarily to neutral variation in allele frequencies or directional selection on the loci or linked regions of the genome. The program fdist2 (Beaumont and Nichols 1996) was used as in Murray and Hare (2006). Simulations of neutral F_{ST} under mutation–drift equilibrium were run using the infinite alleles model for 100 demes (islands), five populations, sample sizes of 50, 50,000 realizations (loci), and an expected F_{ST} equivalent to the global F_{ST} value estimated above ($F_{ST} = 0.035$). The medians and upper and lower 99th quantiles (percentiles) were calculated for the simulated F_{ST} data, and the simulated and actual per locus F_{ST} data were plotted against the inferred heterozygosity (supplementary appendix S4).

Assignment tests were performed in AFLPOP 1.1 (Duchesne and Bernatchez 2002) using all 163 AFLP loci. In this analysis, no correction was made for dominance; each individual's genotype consisted of the presence/absence of the

Table 2 AFLP final amplification primer combinations used on five *H. rufescens* samples

Primer combination	*Mse*I primer	*Eco*RI primer	Approx. size range (basepairs)	Number of markers
1	*Mse*I core + CCC	*Eco*RI core + CCC	110–225	10
2	*Mse*I core + CCC	*Eco*RI core + CGG	150	1
3	*Mse*I core + CCG	*Eco*RI core + CCC	90–310	5
4	*Mse*I core + CCG	*Eco*RI core + CGG	120–375	12
5	*Mse*I core + CTG	*Eco*RI core + ACC	195–425	41
6	*Mse*I core + CTG	*Eco*RI core + ACG	125–225	20
7	*Mse*I core + CTG	*Eco*RI core + AGC	150–350	35
8	*Mse*I core + CTG	*Eco*RI core + AGG	115–300	38

*Eco*RI core sequences consist of 5′-GACTGCGTACCA-ATTC-3′ and *Mse*I core is 5′-GATGAGTCCTGAGTAA-3′

All markers chosen were polymorphic

163 amplified fragments. Each individual was removed in turn from the data set and allele frequencies were recalculated across the loci. Log-likelihoods (lnL) were then estimated for each individual as a member of each candidate population, based on the likelihood that the genotype matched the allele frequency profile of a candidate population better than other candidate populations (with a specified difference in minimum acceptable lnL (lnL_{min})).

An evaluation of the spatial distribution of individuals within populations was performed using the multivariate technique principle coordinates analysis (PCA) option in GenAlEx. A pairwise genetic distance matrix was generated from the binary data file, where the total pairwise distance between individuals is the sum of the pairwise distances for each locus (either one or zero). The PCA was performed directly on the matrix after distances were standardized by dividing each by $(n-1)^{-2}$, where n is the number of pairwise comparisons.

Results

COI

In 483 bp of partial COI sequence obtained for 309 red abalone, 43 haplotypes resulting from 39 individual mutations were observed (Fig. 2; see also S3 for haplotype counts). Representative haplotype sequences were deposited in GenBank under the accessions DQ297507–DQ297549. There was one common haplotype at a frequency of 0.38, and two additional haplotypes at mid-frequency (0.16 and 0.15). The frequencies of the remaining 40 haplotypes were $\leq$0.08. The variation resulted in five amino acid changes within the species (translation based on the invertebrate mitochondrial amino acid code; first codon started at base-pair 3 of our 483 bp of sequence). Non-polar to polar singleton changes occurred twice (Ala1Thr, Phe48Cys), but the remaining three low-frequency base changes did not alter polarity or charge (Ile30Met, Ala91Gly, Ala146Gly).

The mitochondrial data provided little evidence for population genetic structure in global or pairwise comparisons among populations (Table 3). Regardless of grouping, over 99% of variation was within populations rather than among populations or groups (global $\Phi_{ST} = 0.007$). There was no signal of variation between the two Van Damme samples, VD and VDJ. No significant association was found between haplotype and geographic location at any level; the clades appeared to be randomly distributed with respect to population samples.

Microsatellites

See Table 1 for basic statistical information. The compound repeat *Hka*3 locus contained 75 alleles in our sample. The remaining four dinucleotide repeat loci contained 26–36 alleles. There was no evidence for linkage disequilibrium (LD) between any loci in any of the populations after sequential Bonferroni correction at the table-wide $\alpha = 0.05$ level. Significant departures from Hardy–Weinberg equilibrium (HWE) due to heterozygote deficiency were found at *Hka*56 and *Hka*80 (Table 1). Null alleles, non-random mating, inbreeding, or temporal genetic variation of recruits are possible explanations (Miller et al. 2001). Missing genotypes at microsatellite loci were only scored as null homozygotes if the same DNA sample yielded scoreable results at other loci. Only *Hka*56 exhibited a high inferred null allele frequency at 0.31, which did not ultimately affect significance levels in our analyses. Analysis of all microsatellite loci combined revealed global differentiation ($P = 0.002$) but no pairwise divergence (Table 3) and no pattern of isolation by distance. Minor pairwise population divergence between VDJ and HSC was not significant after sequential Bonferroni correction. There was no significant variation in allele frequencies between the two Van Damme samples, VD and VDJ.

AFLPs

A total of 163 polymorphic loci from the eight final amplification primer combinations was scored in a subset of 209 abalone from five populations (CC, 7HC, HSC, MY, and SMI). A reproducibility test on a subset of abalone showed that there was <2% error in independent scoring between runs. There were no fully diagnostic markers (private alleles) for any of the populations. There was no evidence for redundancy among the loci. Uncorrected dominant band allele frequencies for the markers ranged from 0.041–0.994 in the full sample set. Inferred (corrected) allele frequencies at some loci varied by as much as 80% between populations.

Out of 163 markers, 41 (~25%) exhibited significant F_{ST} estimates based on inferred allele frequencies (all $P < 0.001$; data not shown). Only two loci (<2%) that fell outside of the upper 99th quantile in the outlier test of selection were considered outliers potentially under selective pressure. Approximately 4% of variation was between populations in the AMOVA, and estimates of Φ_{ST} indicated a significant level of global divergence among the five populations with patchy pairwise divergence (Table 3). Global differentiation over all five samples was significant as well ($\chi^2 = 1032$, $df = 326$, $P < 0.001$), and nine of ten pairwise comparisons were highly significant (all $P < 0.001$). The tenth comparison (7HC vs. HSC) was borderline significant ($P = 0.055$). In the cladogram, distances were small and no geographic structure was apparent, but the branches were well-supported, suggesting that the populations are genetically distinct (Fig. 3).

Table 3 Global and pairwise sample comparisons for ten samples of *H. rufescens* from nine California sites

Comparison	Exact tests			*F*-statistics				
	COI *P*	μsats *P*	AFLPs *P*	COI Φ_{ST}	F_{ST}	μsats ρ_{ST}	Φ_{ST}	AFLPs Φ_{PT}
Global	0.066	*0.002*	*<0.001*	0.007	0.002	0.001	−0.001	*0.035*
CC & TR	0.154	0.033		−0.008	0.007	−0.006	0.015	
CC & SH	0.355	0.505		−0.010	0.001	−0.002	−0.004	
CC & 7HC	0.040	0.418	*<0.001*	0.052	0.001	0.003	−0.002	*0.058*
CC & VD	0.018	0.013		0.012	0.007	−0.014	0.005	
CC & VDJ	0.024	0.010		0.007	0.005	−0.001	0.015	
CC & SP	0.392	0.075		0.007	0.002	−0.005	−0.005	
CC & HSC	0.462	0.484	*<0.001*	−0.009	0.001	−0.003	−0.003	*0.049*
CC & MY	0.116	0.112	*<0.001*	0.017	0.001	−0.020	0.003	*0.112*
CC & SMI	0.211	0.896	*<0.001*	−0.001	−0.001	−0.010	−0.004	0.022
TR & SH	0.473	0.556		0.001	0.002	0.004	0.008	
TR & 7HC	0.008	0.228		0.036	0.001	−0.008	0.003	
TR & VD	0.009	0.029		0.008	0.003	0.000	−0.001	
TR & VDJ	0.027	0.025		0.015	0.000	−0.002	−0.010	
TR & SP	0.421	0.104		−0.007	0.003	0.003	0.004	
TR & HSC	0.234	0.051		0.006	0.001	0.009	0.002	
TR & MY	0.080	0.282		−0.002	0.003	−0.004	0.003	
TR & SMI	0.111	0.057		0.000	0.004	0.010	0.003	
SH & 7HC	0.084	0.690		0.050	0.001	0.007	−0.003	
SH & VD	0.202	0.254		0.012	0.002	0.000	0.001	
SH & VDJ	0.154	0.092		−0.017	0.000	0.020	0.002	
SH & SP	0.313	0.497		0.018	0.002	−0.007	0.000	
SH & HSC	1.000	0.081		−0.020	0.002	−0.007	0.000	
SH & MY	0.263	0.408		0.020	0.001	−0.011	0.005	
SH & SMI	0.871	0.589		0.002	−0.001	−0.005	0.000	
7HC & VD	0.218	0.064		0.016	0.002	0.007	−0.004	
7HC & VDJ	0.043	0.152		0.032	0.002	0.001	−0.005	
7HC & SP	0.284	0.118		0.011	0.004	0.007	−0.005	
7HC & HSC	0.123	0.781	0.055	0.025	−0.002	0.010	−0.004	0.011
7HC & MY	0.315	0.479	*<0.001*	−0.002	0.006	0.002	−0.001	0.034
7HC & SMI	0.597	0.406	*<0.001*	−0.007	0.001	0.017	−0.002	0.021
VD & VDJ	0.243	0.387		−0.001	−0.001	0.004	−0.006	
VD & SP	0.165	0.077		0.018	0.007	−0.002	0.000	
VD & HSC	0.117	0.003		0.010	0.004	−0.001	−0.002	
VD & MY	0.979	0.116		−0.020	0.007	−0.015	−0.002	
VD & SMI	0.492	0.114		−0.009	0.004	−0.007	0.000	
VDJ & SP	0.066	0.076		0.024	0.002	0.010	−0.002	
VDJ & HSC	0.254	0.251		−0.015	0.001	0.026	−0.004	
VDJ & MY	0.337	0.212		0.012	0.002	0.005	−0.006	
VDJ & SMI	0.156	0.012		0.002	0.003	0.016	0.002	
SP & HSC	0.235	0.223		0.011	0.002	−0.002	−0.005	
SP & MY	0.637	0.590		−0.002	0.001	−0.013	0.002	
SP & SMI	0.504	0.410		−0.003	0.002	−0.009	−0.001	
HSC & MY	0.125	0.664	*<0.001*	0.018	0.001	−0.012	−0.003	0.055
HSC & SMI	0.842	0.070	*<0.001*	−0.014	0.002	−0.004	−0.001	0.021
MY & SMI	0.299	0.066	*<0.001*	−0.008	0.000	−0.015	0.000	*0.072*

Samples at Van Damme taken in 1999 (VD) and 2001 (juveniles <50 mm shell length, VDJ)

Table includes *P*-value results from exact tests of differentiation and estimates of parameter(s) Φ_{ST} for mitochondrial COI; F_{ST}, ρ_{ST}, and Φ_{ST} for nuclear microsatellites (μsats); and Φ_{PT} for AFLPs

Significant *P*-values and parameter estimates after sequential Bonferroni correction at the $\alpha = 0.05$ level in *italics*

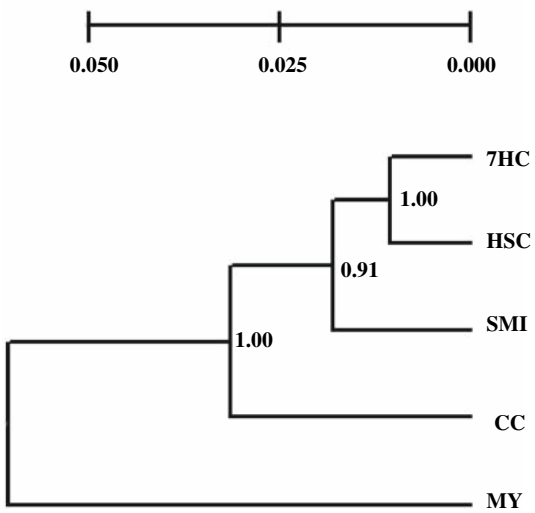

Fig. 3 UPGMA dendrogram of AFLP genotyped *H. rufescens* samples. Branch lengths according to Nei's distance (1972). Branch support values based on 5,000 bootstrap replicates

In assignment tests, only MY was successfully reassigned at a rate of 0.792 (Fig. 4). Even when the $\ln L_{min}$ was dropped to $\ln L_{min} = 0.001$, the remaining populations only reassigned at rates of between 0.257 (SMI) and 0.580 (CC). The success of these global assignment tests was generally low, but specific pairwise assignment tests with the MY population fared better. For example, comparing the two southern populations, SMI versus MY, yielded correct assignment of 0.742 and 0.833 of the individuals, respectively. In principle coordinates analysis, despite great overlap among the populations, MY was closely clustered in space, consistent with the results of the assignment tests (Fig. 5).

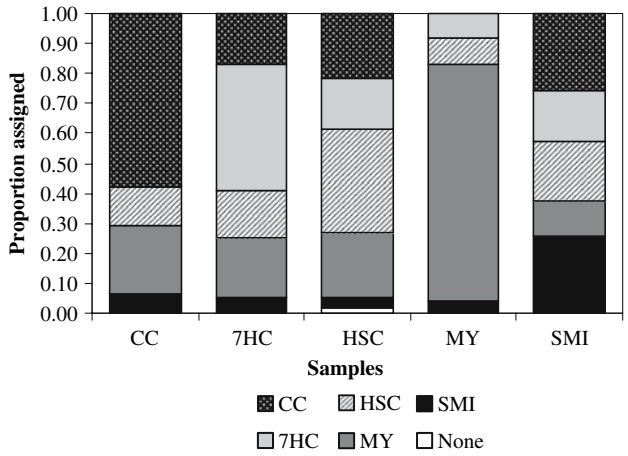

Fig. 4 Assignment test allocation proportions for AFLP genotyped *H. rufescens* samples. Allocation rates based on a minimum log-likelihood difference of 0.001. *Shading* in *vertical bars* represents proportion of individuals in a population or group assigned to a population or group

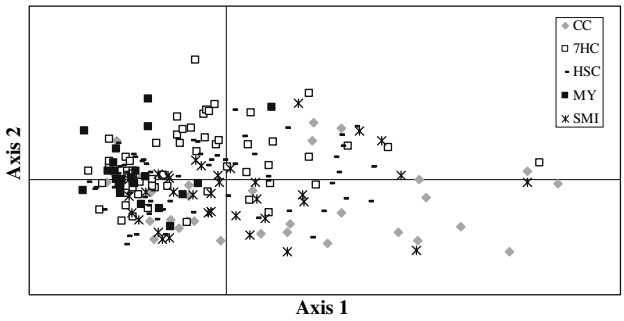

Fig. 5 Principal coordinates analysis on pairwise genetic distances between *H.rufescens* individuals. All 163 AFLP markers were used in analysis. Plot offers maximum separation among populations. Coordinate axes 1 and 2 are shown

Although available resources limited the number of abalone populations subjected to AFLP analysis, the higher level of population differentiation observed for AFLP markers relative to COI and microsatellites was not a result of the difference in population sampling. The five populations sampled for AFLPs spanned the full geographic range of the study, and when COI and microsatellite data sets were reanalyzed in the truncated set of five populations, global divergence at COI was not significant ($\Phi_{ST} = 0.010$). Global divergence at the microsatellite loci was also not significant ($\rho_{ST} = -0.003$).

Discussion

In studies of species with high potential gene flow such as broadcast spawning marine invertebrates (Strathmann 1985; Hedgecock 1994; Waples 1998), the power of genetic analyses for detecting population structure can be increased by using both various types and greater numbers of genetic markers. Prior, small-scale projects typing a nuclear microsatellite locus (Kirby et al. 1998), mtDNA sequence, and allozyme loci (Burton and Tegner 2000) in red abalone found no significant genetic differences among populations, consistent with high interpopulation gene flow. Results presented here, including more extensive analyses of mtDNA sequence (expanded sample size compared to previous studies) and microsatellite loci (increased number of loci and increased sample size), are consistent with the earlier work: the hypothesis of population homogeneity on a broad geographic scale could not be rejected based on these genetic markers (although global microsatellite differentiation hinted at the possibility).

In addition to mtDNA sequence and microsatellite data, we present the results of the first AFLP study on red abalone. Despite their widespread use on other organisms (e.g. plants and bacteria), application of AFLP methods to animal populations in general and marine animal populations

in particular has been limited (Bensch and Åkesson 2005). However, several recent investigations suggest that AFLP methods provide an efficient approach to increasing the power of genetic analyses of population structure (Baus et al. 2005; Brazeau et al. 2005; Shank and Halanych 2007; Sønstebø et al. 2007; Weetman et al. 2007). We found that AFLP protocols offered a reliable and efficient method for scoring genetic variation at 163 loci, presumably spread genome-wide (Vos et al. 1995; Mueller and Wolfenbarger 1999; Campbell et al. 2003; Avise 2004). Using AFLPs, we expanded the number of markers in the red abalone by nearly 70-fold with respect to earlier studies and gained power for detecting significant population genetic divergence. In fact, 25% of the AFLP markers revealed significant differentiation among populations.

The nature of different genetic markers dictates that different evolutionary forces may differentially impact each marker's respective levels of diversity within and between populations. For example, because mtDNA is maternally inherited, it experiences reduced effective population size (potentially increasing random genetic drift) and sex-specific gene flow (only female dispersal impacts mtDNA connectivity measures). Hence, it is not surprising that several studies have found that different genetic markers give contradictory results with regard to population structure (e.g. Karl and Avise 1992; Shank and Halanych 2007). Our finding of population differentiation at AFLP loci but not with other molecular markers is consistent with some recent studies of marine organisms such as the hydrothermal vent tubeworm *Riftia pachyptila* (Shank and Halanych 2007) and the shrimp *Crangon crangon* (Weetman et al. 2007). It is important to note that AFLPs do not always find more pronounced population differentiation than other markers. Gomez-Uchida et al. (2003) found significant differentiation among *Cancer setosus* crab populations with allozymes, which was not observed in parallel AFLP analyses.

As individual markers, one significant weakness of AFLPs is that they are dominant markers (dominant homozygotes cannot be distinguished from heterozygotes). Although dominant markers convey less information than co-dominant markers, Campbell et al. (2003) have shown that the ability to score large numbers of markers can compensate for the information lost at each dominant locus. A consequence of the dominant nature of AFLPs is that the data can be analyzed in different ways. Using an assumption of Hardy–Weinberg equilibrium (HWE), the frequency of the recessive "allele" (no band) can be calculated as the square root of the homozygous recessive frequency. These inferred frequencies can then be used in standard population genetic analyses. Similar approaches are common in analyses of microsatellite null alleles and were used in our own microsatellite analyses.

Is the assumption of HWE defensible? Although many marine populations show deviations from HWE at one or more gene loci, all three allozyme loci scored by Burton and Tegner (2000) in red abalone were found to be in HWE and three of five microsatellite loci scored in this study were in equilibrium (deviations at the other two are best explained by the presence of null alleles). Based on these observations, we do not believe that the assumption of HWE used in calculating AFLP allelic frequencies invalidates our general conclusions. Alternative methods of AFLP analysis do not make the assumption of HWE, and, instead, treat each AFLP band as a simple dominant (present/absent) character. This approach underlies the assignment tests and PCA analyses, which also showed evidence of population differentiation, especially with regard to the MY sample.

A second issue is that inconsistencies in banding intensity in this PCR-based method could potentially lead to inaccuracies in the data matrix. Where variation in banding intensity proves to be a significant issue, any suspect loci can simply be eliminated from the data set. For example, in the shrimp (*Crangon crangon*), relatively few AFLPs could be reliably scored (Weetman et al. 2007). In our own reproducibility studies, scoring error was <2%, suggesting that such problems were unlikely to impact subsequent analyses and interpretation. Furthermore, recent work on abalone using AFLPs found that the preponderance of loci scored showed normal segregation in families (Liu et al. 2006), again attesting to the validity of AFLP studies in abalone.

These AFLP data provide the first evidence for genetic differentiation among populations of red abalone along the California coast. F_{ST} estimates and exact tests of population differentiation were significant even after adjustment for multiple tests, clearly rejecting the hypothesis that red abalone populations are genetically homogeneous across the sampled locales. However, no easily discernable geographic patterns (e.g. isolation by distance) were apparent. The five population samples analyzed lie within the Oregonian biogeographic province and the transition zone between the Oregonian and Californian provinces. Cape Mendocino is a reputed phylogeographic break for marine species (Edmands 2001; Cope 2004), and our samples from north of the Cape showed significant divergence from the two northern California populations sampled south of the Cape (7HC and HSC, the only pair of populations that could not be distinguished by the AFLP markers).

Genetically, MY was the most distinctive sample. The observed genetic differentiation of the MY sample is consistent with observations by Marko (1998), Dawson and Jacobs (2001), and Dawson (2001), suggesting that the Monterey Peninsula is another phylogeographic break reinforced by hydrographic characteristics different from the open coast (see http://www.newark.cms.udel.edu/~brucel/

realtimemaps/) that could result in larval retention (Sponaugle et al. 2002). In addition, while genetic homogeneity is maintained in the larger populations, a small population such as that found at MY (see below) would be more responsive to differentiation due to random genetic drift. Although size-age relationships in abalone are not straightforward (Leighton 2000), the divergence at MY may also be due to narrower cohort structure (i.e. fewer year classes). The MY sample consisted of individuals of <150 mm shell length (see supplementary Table S2), whereas several other samples encompassed larger size ranges. Unfortunately, our lack of cohort sampling prevented assessment of stochastic events such as sweepstakes recruitment (Hedgecock 1994). There was no difference between our two cohorts from Van Damme (VD, VDJ), but the result cannot be extrapolated sample range-wide.

Despite its geographic distance, our SMI sample was genetically more similar to the northern California populations than to MY. San Miguel Island is south of Point Conception, a biogeographic and disputed phylogeographic break (see Burton 1998; Dawson 2001). However, faunal/floral survey evidence suggests that SMI, on the outskirts of the Southern California Bight (SCB) and in the path of the south-flowing California Current, is ecologically more similar to northern California cold water habitats than warmer waters in the SCB (Clark et al. 2005; see also http://www.nps.gov/archive/chis/rm/HTMLPages/MarineResources.htm). Given the data in hand, we can only speculate that the SMI population has experienced relatively recent or on-going recruitment from northern populations. Further, surveys have indicated high natural abundances of red abalone throughout northern California and on SMI (populations perhaps less responsive to random genetic drift), while many central and southern California populations were historically less abundant and have been severely depleted or extirpated in recent decades due to overfishing and, at some locations, sea otter predation (CDFG 2005).

Patterns of connectivity can be obscured if the genetic markers employed are subject to selection (e.g. Karl and Avise 1992). We considered the possibility that the 41 markers exhibiting significant divergence among the red abalone populations were subject to higher levels of selection than the bulk of markers showing no genetic structure (see Lewontin and Krakauer 1973). To address a similar question, Murray and Hare (2006) used a mutation–drift equilibrium model to test for selection on AFLP loci across a secondary contact zone in two *Crassostrea virginica* oyster populations. Their results indicate that AFLPs can efficiently characterize the genomic distribution of F_{ST} and that equilibrium models can be used to evaluate outliers experiencing divergent selection. Our results using this same model suggested that <2% (2 of 163) of our AFLP loci may be under directional selection (i.e. the majority of the allelic frequency variation fits the neutral model), similar to that found in other studies (Wilding et al. 2001; Campbell and Bernatchez 2004; Murray and Hare 2006). Even those two markers, however, could represent extremes in a broad distribution of neutral variation that is not well sampled (Murray and Hare 2006), a possible consequence of insufficient sampling of loci. Assignment test results also suggested that we may have genotyped too few AFLP loci to successfully identify source populations for individual abalone. Unfortunately, despite considerable effort, we were unable to obtain population samples from additional southern California sites to further investigate the possibility that region-wide assignment tests might be more successful in central and southern California where geographic distances among extant populations might be greater than in northern California. Campbell et al. (2003) pointed out that larger numbers of loci are required when larger numbers of weakly differentiated populations are used in the tests. Further work is needed to explore the possibility that additional AFLP markers will enhance assignment success. Although we have substantially increased our total marker set for this species, an additional 100+ markers may be required to successfully assign a high proportion of individuals to the proper population (Campbell et al. 2003).

Understanding the genetic structure of a long-lived species with population admixture can be facilitated by sampling specific recruitment events or size/age cohorts (Flowers et al. 2002; Hellberg et al. 2002). Unfortunately, we were not able to obtain such samples for red abalone; all of our samples were composed of multiple year classes and they were collected in different years. Few abalone larvae are ever seen in the field (Prince et al. 1987) and juvenile abalone are cryptic, hiding under other biota (e.g. urchins) or rocks and in crevices (Leighton 2000; CDFG 2005). Abalone recruitment modules (ARMs) and baby abalone recruitment traps (BARTs) consisting of cinder block "habitat" in a wire cage have been set up along the coast of California at several locations to monitor settlers (Davis et al. 1997), but they have met with limited success due to presumed recruitment failure and not failure of the devices (Rogers-Bennett et al. 2004; see also DeFreitas 2003). Even collecting adult samples was difficult in some locales; our total sample size south of SFB is small as most extant populations south of SFB have extremely low abundances (CDFG 2005). In fact, accidental resampling of individuals is a concern (L Rogers-Bennett pers. comm.); several red abalone from the Monterey area where samples have been collected over multiple years exhibited identical multilocus microsatellite genotypes (data not included in these analyses).

Recent evidence from other marine species indicates that homogeneity among populations of species with planktonic larvae may not be as common as previously thought, and

long-distance larval dispersal may be sporadic at best (Warner and Cowen 2002; Gilg and Hilbish 2003; Veliz et al. 2006; see also Sponaugle et al. 2002). World-wide, several abalone species have shown evidence of genetic subdivision. The blacklip abalone (*H. rubra*) from southern Australia exhibited divergence between populations from Victoria and Tasmania (Ward and Elliot 2001), and the perlemoen (*H. midae*) from South Africa shows regional divergence east and west of Cape Agulhas (Evans et al. 2004). Most recently, the tropical abalone (*H. asinina*) was shown to be divided into populations from the Gulf of Thailand and the Andaman Sea (Tang et al. 2005). Previous studies of abalone species along the Pacific coast of North America have revealed considerably less population divergence. Using allozyme electrophoresis, Hamm and Burton (2000) and Chambers et al. (2006) have found some evidence for population structure in California black abalone (*H. cracherodii*). Withler et al. (2003) found little evidence for genetic structure in microsatellite allele frequencies in northern abalone (*H. kamtschatkana*), and, as discussed above, Burton and Tegner (2000) found no evidence for population divergence in the red abalone. As such, this study presents the first evidence of significant population differentiation in red abalone, but continued research is needed to further examine the spatial scale and temporal stability of genetic subdivision in this species.

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